Allagochrome. I. Properties, Purification, & Assay Procedures[1, 2]

Helen M. Habermann

Department of Biological Sciences, Goucher College, Towson, Baltimore 4, Maryland

Allagochrome[3] is a water-soluble pigment which has been extracted from the leaves of a number of species of higher plants. Attempts to purify and characterize allagochrome have revealed that the apparently uniform fraction obtained following electrophoresis and chromatography on sephadex has at least three distinct but tightly bound components: 1) a chromophoric group absorbing in the red with a peak at approximately 670 mμ, 2) copper, and 3) a fluorophoric group emitting at 460 mμ when excited by ultraviolet light. The most conspicuous of the three is, of course, the chromophoric group which is responsible for the characteristic blue-green appearance and the striking color changes of allagochrome solutions in response to oxidation or reduction and pH.

When allagochrome was first described (3), no records had been found in the literature of this or comparable compounds from leaves. Since that time it has been learned that in 1950 Kozlowski, working in Hill's laboratory, had reported the formation of a green oxidation-reduction indicator in plant extracts (7). Also, Kahn, Dorner, and Wildman in the mid to late 1950's extracted and attempted to purify a green compound from tobacco and artichoke which appears to have been allagochrome. The attempts of the latter workers to purify and characterize their green stuff were not completely successful and were never published (private communication, Dr. R. W. Dorner).

A copper protein extracted from *Chlorella ellipsoidea* with properties similar to allagochrome has recently been reported by Katoh (6). These two pigments are not identical spectrophotometrically, however, and the copper protein from Chlorella is not autoxidizable. All assays of *Chlorella pyrenoidosa* for allagochrome have been negative. Substances with similar spectra and electrochemical properties have been extracted from spinach chloroplasts by Takamatsu and from chloroplasts of *Chrysanthemum coronarium* by Katoh (6). It is quite possible that the preparation from Chrysanthemum is allagochrome because we have found that Chrysanthemum species are an excellent source of this pigment.

The absorption spectrum of allagochrome strongly resembles action and absorption spectra of the red-absorbing form of phytochrome (1). According to present information about these pigments, there is no relationship between them aside from the resemblance in color.

It is the purpose of this paper to summarize 1) the progress in attempts to purify allagochrome, 2) the methods which have been developed to assay for this component of leaves, and 3) some speculations about its chemical nature.

Methods

I. *Extraction and Purification.* Freshly harvested leaves are ground in a Waring blendor with glycine-NaOH buffer (pH 9.5, 0.05 M glycine, 0.02 M NaOH; 30 ml buffer per 10 gm leaves). The brei is filtered to remove large particles. Subcellular particles are removed by centrifugation at 4° and 30,000 × *g* in a refrigerated centrifuge. Allagochrome can be removed from the resulting crude extract by precipitation with acetone (fraction between 45 & 90 % acetone). Despite certain advantages of acetone precipitation (such as concomitant removal of traces of plastid pigments & concentration of allagochrome) we have omitted this step in purification because the precipitation of pigment is not quantitative and it is difficult to remove traces of acetone. The next step in purification (electrophoresis) is now used to remove the remaining chlorophylls and carotenoids. The crude extract is fed onto the paper curtain of the electrophoresis apparatus (Spinco model CP) at a rate of 7 to 10 ml per hour. Glycine-NaOH buffer of the same concentration as used for extraction serves as the electrolyte. Plastid pigments are adsorbed on the paper curtain in the region of application. The relative amounts of contaminants removed in this way determine how long the curtain can be used before their accumulation begins to interfere with the resolution of separating components of the crude allagochrome preparation. Generally, curtains have to be replaced every 4 to 5 days under these circumstances, whereas continuous operation for several weeks is possible if plastid pigments have been removed prior to electrophoresis. Migration toward the positive electrode under an applied potential in the range of 550 to 800 volts is rapid and the apparatus is adjusted by backwash of the separating fractions so that allagochrome is collected on the

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[3] The term allagochrome was derived from the Greek words *allago* = changing in form, and *chromos* = colored substance.

positive electrode side at the bottom of the curtain. At this stage of purification, relatively large amounts of pigmented components other than allagochrome are separated and at least two yellow fractions are partially removed from the allagochrome preparation.

The properties of some of the yellow pigments obtained by electrophoretic purification of crude extracts of Helianthus leaves and seeds have been summarized in an earlier paper (4). Comparable fractions have more recently been extracted from Chrysanthemum, Phytolacca, and Ligustrum leaves. Techniques for purification and assay of these yellow pigments have not yet been developed. Studies of the distribution of allagochrome (2) and purification of allagochrome from several sources have indicated, however, that such yellow components are closely associated with allagochrome wherever it occurs. Extracts from species which have no measurable absorption peaks in the red portion of the spectrum often contain large amounts of yellow pigments with peaks in the 400 to 500 mμ region of spectra measured against aliquots reduced with sodium hydrosulfite as blanks (see assay procedures below).

After electrophoresis, traces of the yellow components still remain in the allagochrome fraction. Allagochrome is further purified by passage through a column of No. 75 grade regular sephadex. The yellow components in part precede and in part follow allagochrome on sephadex, indicating a gradient in molecular size with allagochrome intermediate in size between the two yellow pigments.

Fractions which have become diluted by electrophoresis and chromatography are concentrated by lyophilization. Preparations of allagochrome and of several of the associated yellow pigments from three sources (*Helianthus annuus*, Chrysanthemum sp. & *Ligustrum japonicum*) have been purified by repeated electrophoresis and chromatography on sephadex. Lyophilization of such preparations after the last passage through sephadex has yielded crystalline solids.

II. *Assays for Special Components.* A quantitative assay for copper modified from that described by Humphries (5) is used for analysis of allagochrome preparations. Phosphorus content is determined by a method described by Pirson and Kuhl (8).

III. *Assays for Allagochrome.* Leaves (usually 1–2 g) weighed immediately after harvesting are ground with cold glycine-NaOH buffer either by hand in a chilled mortar or in a small Waring blendor. The homogenate is transferred with several washings of buffer to a graduate cylinder, diluted to the desired volume (according to the size of the sample) and then centrifuged at 4° for 15 minutes at 30,000 × *g* (International HR-1 refrigerated centrifuge, 8-place 10.5 cm radius head, 17,500 rpm). Aliquots of the clear supernatant are transferred to a pair of matched cuvettes and a few crystals of sodium hydrosulfite are added to one or reduce the allagochrome. The absorption spectrum of the sample is measured in a Beckman DK-2 recording spectrophotometer using the reduced aliquot as blank. The optical density of the peak in the red portion of the spectrum is proportional to the concentration of oxidized allagochrome in the sample. The plastid pigments, which are the major colored contaminants, do not change color on addition of the reducing agent, are present in equal concentrations in sample and blank, and thus do not contribute to the recorded optical density. Reduced allagochrome does not absorb in the red portion of the spectrum (figs 2a, 2b, & 2c).

Optical densities of solutions containing known weights of allagochrome per ml of solution have been determined for purified preparations from Helianthus, Chrysanthemum, and Ligustrum leaves. These determinations were made either by drying 5 to 10 ml aliquots of samples of known optical density to constant weight at 95° or by dissolving weighed amounts of lyophilized samples.

Allagochrome Value as a Unit of Pigment Concentration. Although purified preparations of allagochrome from several species have identically shaped spectra, there is considerable variation between preparations from different species in the weight of pigment needed per ml for solutions of given optical density. Such differences make it impossible to utilize concentration optical density relationships determined for a given species for calculation of weights of allagochrome present in samples from other species. For this reason, the following expression (which reflects the concentration of the pigment but makes no assumptions about weights of materials associated with the chromophore) has been used as a measure of relative allagochrome concentration:

$$\text{Allagochrome value} = \frac{\text{OD (at red max, 1 cm)} \times \text{vol of homog (ml)}}{\text{Weight of sample (g)}}$$

IV. *Changes in Absorption Spectra With pH.* In order to determine whether the chromophores of purified allagochrome from different sources are identical, absorption spectra of the oxidized and reduced forms of Helianthus, Chrysanthemum, and Ligustrum allagochrome were measured over a range of pH between 2 and 13. For measurement, 1 ml of concentrated allagochrome solution was mixed with 9 ml of buffer. Absorption spectra of the oxidized pigments were measured against water plus buffer blanks in a Beckman DK-2 recording spectrophotometer. A few crystals of sodium hydrosulfite were added to both cuvettes and spectra of the reduced pigments were measured immediately after measurement of the oxidized form. See figure legends (1, 2a, 2b, 2c) for pigment concentrations.

V. *Fluorescence Spectra.* Excitation and fluorescence spectra of Helianthus, Chrysanthemum, and Ligustrum allagochrome were measured with an Aminco-Bowman spectrofluorometer. Samples of known concentration (wt per vol) dissolved in water were used. Variation of wavelength of excitation and fluorescence maxima and differences in relative intensity of fluorescence with pH were determined

either by making test solutions alkaline or acidic by adding a drop of concentrated NH_4OH or HCl, or by preparing solutions in the buffer series used for measurement of changes in absorption spectra with pH.

Results & Discussion

I. *Extraction and Purification.* The difficulties of separating non-specific protein and contaminating yellow pigments from allagochrome preparations have been the most persistent problems of purification. The presence of protein in crude preparations makes it difficult to determine whether allagochrome is indeed a pigment-protein complex or whether the association of pigment with protein is a consequence of preparative techniques. On purification, there is a steady decrease in absorbance in the ultraviolet relative to peak height in the red region of the spectrum as well as increased stability towards protein denaturing agents. (See note added in proof.) Spectra of oxidized Ligustrum allagochrome illustrated by figures 1 and 2c show typical ratios of peak heights in the ultraviolet and visible for the most highly purified preparations.

The procedures for extraction and purification described above were developed for preparations from *Helianthus annuus.* They seem to be equally effective for extracts from all other species which have thus far been purified. In the following sections similarities and differences between preparations from several sources will be noted and reasons will be cited for suggesting that the chromophores are the chemically consistent constituent of the allagochrome complex. Only the characteristic of color has been used to identify and estimate the concentration of allagochrome.

II. *Weight per Unit Volume and Optical Density.* Table I summarizes the relationship between concentration expressed as weight per unit volume of solution and optical density at the red absorption peak for preparations of allagochrome from *Helianthus annuus,* Chrysanthemum sp., and *Ligustrum japonicum.* The large differences between samples from different sources indicate that there are considerable differences in the concentration of chromophores within the allagochrome complex and that these differences are probably species-specific.

Table I

Relationship between Concentration & Optical Density for Allagochrome Preparations from Helianthus, Chrysanthemum, & Ligustrum

Sample	mg allagochrome per ml solution with OD of 1	$E_1^{1\%}$ cm
Helianthus		
Liquid samples of known optical density, dried to constant weight	2.48 ± 0.21*	4.03
Lyophilized samples dissolved in H_2O	2.72 ± 0.14	3.68
Chrysanthemum		
Lyophilized samples dissolved in H_2O	0.77 ± 0.03	12.99
Ligustrum		
Lyophilized samples dissolved in H_2O	17.23 ± 1.62	0.58

* Mean ± standard deviation of mean. Optical densities measured in a Beckman DK-2 recording spectrophotometer, 1 cm cuvettes.

III. *Special Components.* Copper has been found in allagochrome preparations at all stages of purification. Although phosphorus has also been shown to be present, its concentration varies with the procedures used for purification. All tests for iron were negative.

A. *Copper.* Although there are relatively large

Table II

Summary of Determinations of Copper in Allagochrome

Sample	Number of determinations	μg Cu per ml solution with OD of 1
A. *Helianthus*		
1. Electrophoretically pure	2	0.479 ± 0.014*
2. Electrophoretically pure	6	0.205 ± 0.023
3. Electrophoretically pure, once rapidly through sephadex, also used for phosphate analyses	3	0.302 ± 0.119
4. Electrophoretically pure, once rapidly through sephadex	6	0.283 ± 0.037
5. Electrophoretically pure, once rapidly through sephadex, concentrated & dialyzed	3	0.228 ± 0.036
6. Electrophoretically pure, once slowly through sephadex, also used for phosphate analyses	2	0.468 ± 0.087
7. Electrophoretically pure, once slowly through sephadex, fractions on either side of sample 6	1	0.406 ...
8. Electrophoretically pure, twice rapidly through sephadex	6	0.195 ± 0.049
B. *Chrysanthemum*		
1. Electrophoretically pure	6	0.065 ± 0.009
2. Electrophoretically pure	12	0.067 ± 0.012
3. Electrophoretically pure	12	0.052 ± 0.007
4. Electrophoretically pure, once rapidly through sephadex	6	0.082 ± 0.018
5. Electrophoretically pure, once rapidly through sephadex	8	0.056 ± 0.015
C. *Ligustrum*		
1. Electrophoreticaly pure, once through sephadex, also used for phosphate analyses	5	2.29 ± 0.41

* Results expressed as mean ± standard deviation of mean. Samples with comparable preparative histories are listed separately to indicate variability of results.

variations in copper content of allagochrome preparations from different species, values are consistent for samples from any given species and copper concentration relative to color does not change appreciably with purification (table II). It is present in allagochrome preparations from all sources which have been tested. Its variable concentration relative to optical density in samples from different sources implies that the color of allagochrome cannot be a consequence of copper content alone.

B. *Phosphorus.* The phosphorus content of allagochrome is extremely variable and depends on the methods of purification (table III). Most samples

Table III

Summary of Determinations of Phosphorus in Allagochrome

Sample	Number of determinations	μg P per ml solution with OD of 1*
Helianthus		
Electrophoretically pure, once rapidly through sephadex	16	0.270 ± 0.020
Electrophoretically pure, once slowly through sephadex	3	0.014 ± 0.010
After repeated electrophoresis & chromatography on sephadex	5	0.038 ± 0.004
Chrysanthemum		
Electrophoretically pure, once through sephadex	12	0.029 ± 0.003
After repeated electrophoresis & chromatography on sephadex	10	0.012 ± 0.002
Ligustrum		
Electrophoretically pure, once through sephadex	10	3.68 ± 0.36

* Mean ± standard deviation of mean.

are purified by relatively rapid passage through sephadex (10–100 ml per hr). If the flow rate is extremely slow (ca. 10 ml per day) the phosphorus content of the effluent allagochrome is reduced to about 1/20 of rapidly purified samples. It appears, then, that phosphorus must be regarded as either a contaminant or a loosely bound component of the allagochrome complex. The latter seems more probable at the present time, although removal of contaminating yellow pigments (which generally contain relatively larger amounts of phosphorus) could at least in part account for the differences in phosphorus content of allagochrome samples with different preparative histories.

IV. *Allagochrome Assays.* The assay for allagochrome is simple and direct yet subject to several kinds of limitations:

A. Allagochrome values can be used to calculate absolute concentrations only in those cases where the relationship between weight of pigment and optical density of solution has been established. Even with highly purified preparations, however, there is no guarantee that a large proportion of the dry weight is not the result of complex formation during extraction and purification. Thus allagochrome values are measures only of relative chromophore concentrations and cannot be extrapolated to molar concentrations or absolute weights.

B. Assays of a wide variety of plant materials have demonstrated that many species of higher plants contain allagochrome. The crude allagochrome extracts are not always spectrophotometrically identical, however. Although the shapes of absorption spectra and responses to reducing agents or changes in pH are typical, there can be wide variation in the wavelength at which the characteristic peak and shoulder occur. Crude extracts of Ligustrum allagochrome exhibit one of the extreme cases of peak position (630 mμ) but this shifts to 670 on purification. Peak positions for all species testing positively have been listed in (2) (also see table IV). Such variations in spectra are probably akin to the much discussed peaks of chlorophyll a spectra in vivo which yield only chlorophyll a on extraction. They appear in response to factors in the chemical environment of crude extracts. These differences are not the result of pH changes during extraction.

C. The accuracy of allagochrome values is dependent on the maintenance of oxidizing conditions and on the extent of extraction of samples. The extent of extraction of allagochrome from Helianthus leaves has been estimated by re-extracting the material precipitated during centrifugation of the initial tissue homogenate. These determinations showed that over 90% of the total extractable allagochrome was present in the first supernatant fraction. The extent of extraction may vary with the species of plant. The great solubility of the pigment in the glycine-NaOH buffer (no limits of solubility in buffer or in water have been observed) and the low yield of a second extraction indicate, however, that the present procedures provide valid estimates of pigment content.

A paper summarizing an extensive survey for allagochrome in representative taxa of vascular plants has been published elsewhere (2). Table IV lists only those species shown to have high concentrations of this pigment. Allagochrome values for species testing positively range from less than 1 to greater than 80. Of the more than 150 species representing over 100 genera which have been assayed, about 45% had measurable concentrations of allagochrome. The absence of measurable red absorption peaks in extracts from many species does not necessarily imply that no allagochrome is present; it might only reflect limitations of measuring instruments or inadequate size of samples. As is the case with so many things, the presence of allagochrome can be demonstrated more easily than its absence.

There is considerable variation in the allagochrome content with position of leaves and plant age. Generally, leaves formed late in vegetative growth

Table IV

Allagochrome Assays—Representative Species with High Allagochrome Content

Order Family	Species	Wavelength of red peak (mμ)	Allagochrome value
	Division Sphenopsida		
Equisetales			
Equisetaceae	Equisetum arvense	670	12.98
	Division Spermatophyta		
	Subdivision Gymnospermae		
Coniferales			
Pinaceae	Tsuga sieboldii	670	20.43
	Tsuga canadensis	670–675	17.14
	Tsuga caroliniana	670	16.08
	Subdivision Angiospermae		
	Class Dicotyledoneae		
Ranales			
Magnoliaceae	Magnolia sp. (deciduous)	668–670	11.50
Berberaceae	Mahonia sp.	635	82.13
	Berberis triacanthophora	670	38.15
	Podophyllum peltatum	670	7.81
Geraniales			
Simaroubaceae	Ailanthus altissima	674–676	8.79
Sapindales			
Aceraceae	Acer rubrum	640–660	13.35
Gentianales			
Oleaceae	Ligustrum Japonicum	635	54.66
	Ligustrum ovalifolium	637–642	33.30
Apocynaceae	Carissa bispinosa	666–670	7.87
Polemoniales			
Solanaceae	Nicotiana rustica	664–673	20.74
Gesneriaceae	Saintpaulia sp.	634–636	6.77
Lamiales			
Labiatae	Glechoma hederacea	640–642	16.38
	Prunella vulgaris	638–642	44.79
	Leonuris cardiaca	660–665	13.30
Rosales			
Rosaceae	Pyracantha coccinea	673	43.99
Crassulaceae	Aeonium cuneatum	670–676	53.37
Celastrales			
Celastaceae	Schaefferia sp.	667–675	12.75
Aquifoliaceae	Ilex aquifolium	664–666	54.24
	Ilex opaca	665	19.87
	Ilex canariensis	668–670	77.56
	Ilex crenata	670	24.82
Umbellales			
Aralaceae	Aralia sp.	665–670	27.17
	Hedera helix	665	28.84
Rubiales			
Rubiaceae	Coffea arabica	670–673	23.12
Caprifoliaceae	Lonicera japonica	660–667	44.27
	Diervilla corvaensis	668–672	17.49
Asterales			
Compositae	Helianthus annuus	673	13.31
	Aster simplex	673	27.59
	Aster novae-angliae	673	54.55
	Chrysanthemum sp.	674–683	44.28
	Eupatorium altissimum	675–678	61.52
	Solidago altissima	672–674	59.68
	Solidago juncea	672–674	37.94

contain more allagochrome than leaves from young plants.

V. *Variation of Absorption Spectra With pH.* Figures 2a, 2b, and 2c illustrate absorption spectra of purified allagochrome preparations from Chrysanthemum, Helianthus, and Ligustrum over a pH range of 2 to 13. Changes in absorption patterns with pH are apparent for both oxidized and reduced forms of the pigment. The close correspondence of positions of absorption maxima and changes in absorption spectra with pH in the visible indicate that the allagochrome chromophores from these species, if not chemically identical, are at least structurally very similar.

Changes in ultraviolet absorption spectra with pH for oxidized Ligustrum allagochrome are shown in figure 1. The solutions are the same as those used to determine spectral changes between 450 and 800 mμ. In contrast to variations in visible spectra,

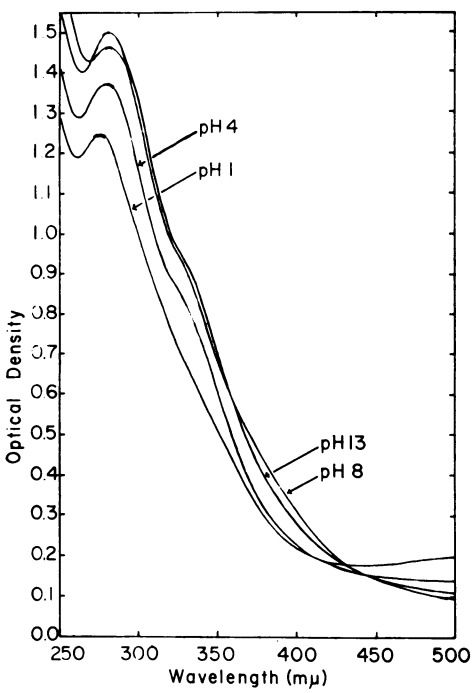
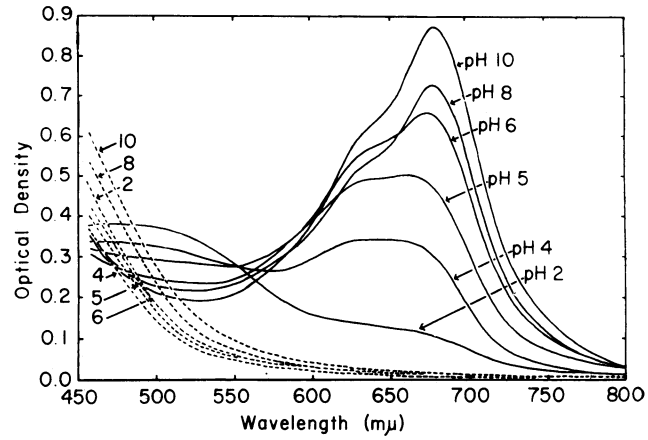
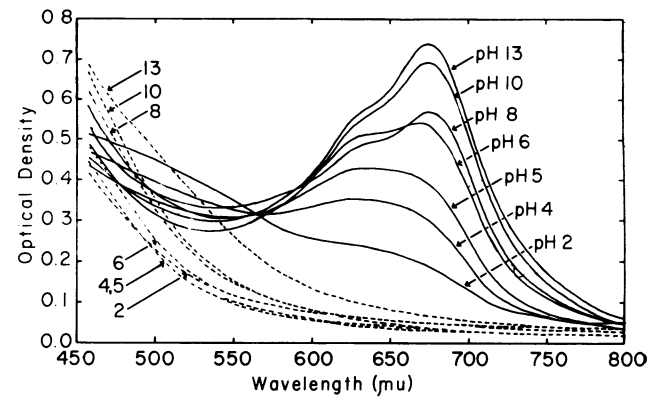
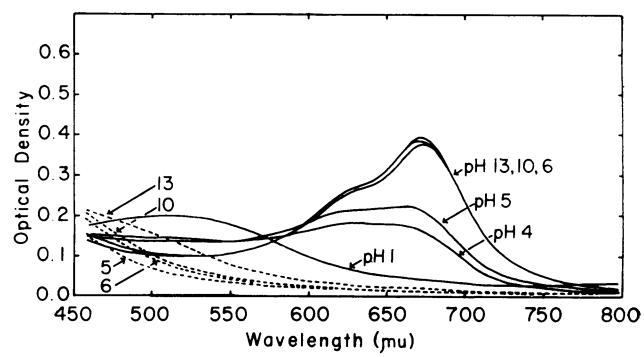


FIG. 1 (*bottom left*). Variation in ultraviolet absorption spectrum of Ligustrum allagochrome with pH. Oxidized pigment measured against solvent blank. Concentration of pigment: 6.4 mg per ml.

FIG. 2 (*right*). Variation in visible absorption spectra of allagochrome with pH. All measurements against solvent blanks. ——— oxidized; - - - - - - reduced with sodium hydrosulfite. a. (*top*) Chrysanthemum allagochrome; concentration: 0.7 mg per ml. b. (*middle*) Helianthus allagochrome; concentration: 2.1 mg per ml. c. (*bottom*) Ligustrum allagochrome; concentration: 6.4 mg per ml.







there are rather minor shifts in ultraviolet absorption patterns with pH. Similar changes in absorption spectra in the ultraviolet have been observed with Helianthus and Chrysanthemum allagochrome preparations.

VI. *Fluorescence Spectra.* The first examination of allagochrome for fluorescence was done at the Carnegie Institution of Washington, Stanford, on an instrument designed for measurement of chlorophyll fluorescence (see discussion, ref 4). At that time only fluorescence due to traces of chlorophyll was observed and this could be eliminated by extraction with chloroform. More recent examination of the fluorescence characteristics of allagochrome with an Aminco-Bowman spectrofluorometer revealed a distinct pattern of fluorescence around λ 460 mμ. Spectra of purified allagochrome from Chrysanthemum, Helianthus, and Ligustrum are shown in figure 4. Absorption spectra of solutions used for these measurements are shown in figure 3. Although activation and emission spectra of allagochrome from all three sources are similar, wave-

Table V

Comparison of Fluorescence Characteristics

Pigment	Solvent	Activation maximum (mμ)	Fluorescence maximum (mμ)	Relative intensity*
Helianthus	H_2O	345–348	458–460	26.5
allagochrome	Dil. NH_4OH	345	458	22
	Dil. HCl	345	465	3
Chrysanthemum	H_2O	350–353	455–458	33
allagochrome	Dil. NH_4OH	350	453	27
	Dil. HCl	355	475	5.5
Ligustrum	H_2O	335	460	31
allagochrome	Dil. NH_4OH	335	458	22
	Dil. HCl	370	480	2
Ligustrum	H_2O	385–390	460–470	...
yellow I		shoulder at 330		

* Arbitrary units.
Concentration of pigment solutions adjusted for approximately equal fluorescence intensity.

lengths of excitation and emission maxima are not identical (table V). All three had the same relative response to pH of solution: highest intensity of fluorescence at neutral and alkaline pH with a sharp drop in intensity in acid solutions.

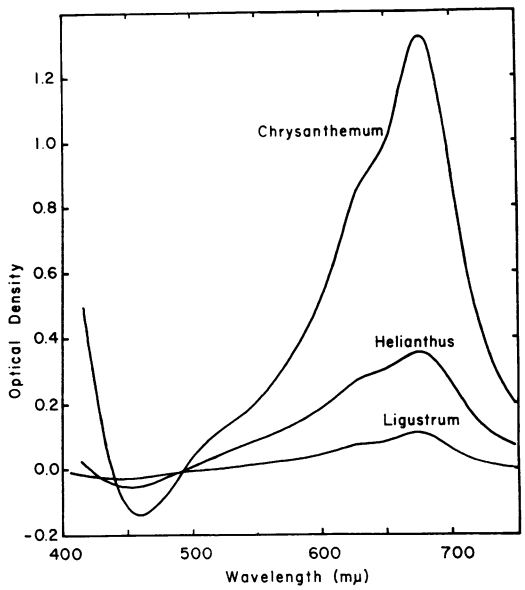
The fluorescence characteristics of several of the yellow pigments separated from allagochrome during purification have also been studied. In contrast to the fluorescence of allagochrome, which is usually not visible except in very dilute solutions due to reabsorption of the emitted light, the fluorescence of the yellow pigments is quite apparent even in concentrated solutions. Emission maxima for the yellow pigments are generally between 450 and 500 mμ (they fluoresce blue, green, or yellow-green when excited by ultraviolet light). There is great similarity between some of the yellow fractions and allagochrome in the position of fluorescence maxima. Their excitation spectra are quite different, however, and they differ in response to pH (table V & fig 5). Although repeated electrophoresis and chromatography on sephadex are necessary for complete removal of traces of the yellow pigments, the fluorescence properties of the most highly purified preparations of allagochrome indicate that the separation was complete so far as this means of detecting the presence of contaminants is concerned. In fact, fluorescence spectra have been utilized as a sensitive test for purity.

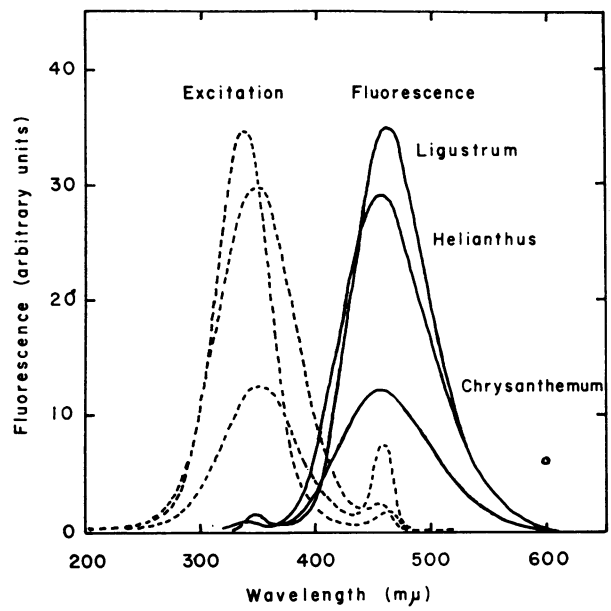
VII. *Some Speculations Concerning the Chemical Constitution of Allagochrome.* Excitation and emission spectra of allagochrome fluorescence resemble those of known pteridines. The similarities between allagochrome (and the associated yellow pigments) and pteridines of known chemical constitution extend beyond the close correspondence of their fluorescence characteristics. Pteridines also exhibit spectral changes on oxidation or reduction and in response to changes in pH. Some are known to be associated with rather large polypeptides.

The chromophore of allagochrome does not fluoresce. Because of the position of the fluorescence peak in a spectral region where there is relatively high absorption by the chromophore, we have to assume the presence of two different light-absorbing groups, one of which resembles the pteridines. There is greater variability in fluorescence spectra of allagochrome preparations from different sources than in their light-absorbing properties. Differences in wavelength of excitation and emission maxima of allagochrome from Helianthus, Chrysanthemum, and Ligustrum are sufficiently large to suggest differences in configuration of the fluorophores.

Even though there is absorption by the chromophore at wavelengths of greatest emission by the fluorophore, the solutions used for these measurements were sufficiently dilute so that only a small part of the differences in relative intensity of fluorescence can be accounted for as reabsorption by the chromophore. If we assume that the observed differences in fluorescence intensity of allagochrome solutions of equal concentration (wt/vol) from different sources reflect differences in fluorophore concentration, the spectra in figure 4 suggest approximately equal fluorophore concentrations in preparations from Helianthus and Ligustrum which are approximately twice that in an equal weight of Chrysanthemum pigment.

Purified allagochrome preparations are relatively stable and have been stored under refrigeration or frozen for over a year without apparent change. The pigment complex is not damaged by light of moderate intensity but high intensity illumination does cause measurable bleaching. Stability increases with purification. Crude preparations tend to become reduced on standing except for the surface layer which is in contact with air. There is no tendency for such autoreduction of purified allagochrome, nor can preparations of high purity be reduced by being made anaerobic through evacuation or flushing with hydrogen or nitrogen. It has not yet been determined whether the reversible reduction of allagochrome in crude preparations is a consequence of specific enzymatic activity or merely due





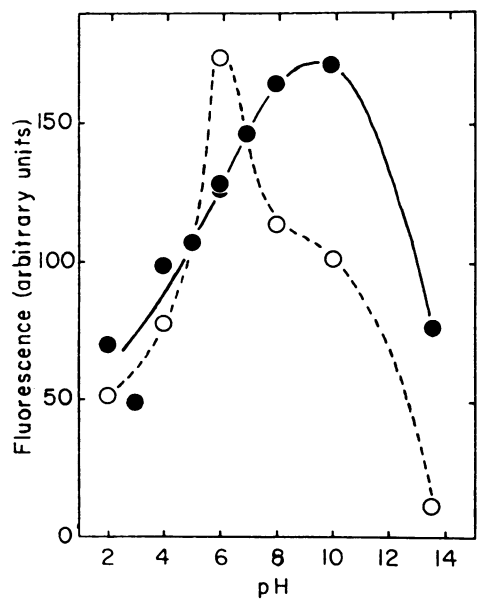


FIG. 3 (*left*). Visible spectra of pigment preparations used for fluorescence spectra. Aliquots reduced with sodium hydrosulfite were used as blanks. Concentrations of pigments: Helianthus: 1 mg per ml; Chrysanthemum: 1 mg per ml; Ligustrum: 2 mg per ml.

FIG. 4 (*upper right*). Fluorescence activation and emission spectra of allagochrome preparations from Ligustrum, Helianthus, and Chrysanthemum. Concentration of all preparations: 0.2 mg per ml. Minor peaks in excitation and fluorescence curves are due to instrument scatter.

FIG. 5 (*lower right*). Variation in relative intensity of fluorescence with pH. Comparison of Ligustrum allagochrome and Ligustrum Yellow I. ———— Allagochrome; ---------- Yellow I.

to the presence of naturally occurring reductants released during grinding. However, it can usually be observed as long as appreciable amounts of the yellow pigments are present.

There is a remarkable similarity between the absorption spectrum of oxidized allagochrome and the action and absorption spectra of the red-absorbing form of phytochrome (1). All other properties of these two pigments are quite different, however. Allagochrome preparations do not exhibit the shifts in absorption spectra on illumination characteristic of phytochrome; phytochrome extracts do not exhibit the color changes on oxidation or reduction and change of pH characteristic of allagochrome. Procedures which are satisfactory for extraction of allagochrome (e.g., grinding in a Waring blendor) result in complete destruction of phytochrome. Species which are good sources of allagochrome are frequently poor sources of extractable phytochrome and vice versa (private communication, Drs. S. B. Hendricks & H. W. Siegelman). At the present time there seems to be no connection between the allago-

chrome and phytochrome pigment systems.

Note added in proof: Preliminary ultracentrifuge and ESR studies done in collaboration with Drs. Ulrich Weiss, Chester W. DeLong, and Marc S. Lewis of the National Institutes of Health have been completed since the preparation of this paper. Calculations of molecular weight based on schlieren patterns observed after prolonged centrifugation at maximum speed (59,780 rpm) indicate that the molecular weight of allagochrome probably lies between 720 and 1400. Certainly the possibility that protein is present in purified allagochrome preparations can be excluded on the basis of ultracentrifuge studies because no high molecular weight components were observed.

ESR spectra of aqueous solutions of purified allagochrome do not show a signal for Cu^{++}. This indicates that copper present in these preparations must be in the Cu^{+} state which cannot be detected by the ESR technique. ESR studies thus confirm the hypothesis that a chromophoric group (probably quinonoid) rather than copper is responsible for color changes associated with reduction or acidification.

The wide discrepancy between molecular weight estimates based on copper content and analytical ultracentrifuge studies places the position of copper as a real constituent of the allagochrome molecule in a questionable status. The results of these recent studies will be published in detail elsewhere.

Summary

I. Allagochrome is a blue-green, water soluble pigment which has been extracted from the leaves of many species of plants. The pigment exhibits reversible changes in color in response to reduction (to yellow) and acidification (to red).

II. This pigment is extracted from leaves by grinding in alkaline buffer. The resulting brei is centrifuged to remove solids and further purification is by continuous flow electrophoresis and chromatography on sephadex.

III. The assay procedure for allagochrome is based on differences in optical density between oxidized and reduced aliquots of crude extracts. Known sources having high concentrations of the pigment are listed.

IV. Comparative studies of purified allagochrome prepared from Helianthus, Chrysanthemum, and Ligustrum included determinations of copper and phosphorus content, changes in absorption spectra with pH, and fluorescence characteristics.

V. Present estimates of molecular size and likely composition, nature of the chromophore and fluorophore and possible relationships with other pigment systems are discussed.

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Literature Cited

1. Borthwick, H. A. & S. B. Hendricks. 1961. Effects of radiation on growth & development. Encyclopedia of Plant Physiology. Springer-Verlag, Berlin. vol XVI: 299–330.
2. Garrick, L. S. & H. M. Habermann. 1962. The distribution of allagochrome in vascular plants. Am. J. Botany 49: 1078–88.
3. Habermann, H. M. 1960. A new leaf pigment. In: Comparative Biochemistry of Photoreactive Systems. M. B. Allen, ed. Academic Press, New York. pp 73–82.
4. Habermann, H. M. 1961. Isolation & function of several newly discovered water-soluble pigments from leaves. In: Progress in Photobiology, Proceedings of the 3rd International Congress on Photobiology. B. Chr. Christensen & B. Buchman, eds. Elsevier Pub. Co., Amsterdam, pp 576–80.
5. Humphries, E. C. 1956. Mineral components & ash analyses. E. Determination of trace elements. IV. Copper. In: Modern Methods of Plant Analysis. K. Paech & M. V. Tracey, eds. Springer-Verlag, Berlin. vol I, pp 493–94.
6. Katoh, S. 1960. A new copper protein from *Chlorella ellipsoidea*. Nature 186: 533–34.
7. Kozlowski, A. 1950. Formation of a green oxidation-reduction indicator in plant extracts. Nature 165: 495.
8. Pirson, A. & A. Kuhl. 1958. Über den Phosphat Aushalt von Hydrodictyon. I. Arch. Mikrobiol. 30: 211–25.